USE OF ANTIBODY Fab′ FRAGMENTS TO REMOVE INTERFERENCE BY RHEUMATOID FACTORS WITH THE ENZYME-LINKED SANDWICH IMMUNOASSAY

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1. Introduction

Recently, we reported a method to remove the nonspecific interference by serum factors with the enzyme-linked sandwich immunoassay [1]. Inclusion of gelatin with a relatively high concentration of salt in the immunoassay mixture was effective to remove the nonspecific interference by serum with the assay.

However a specific interference with the assay system was still observed when sera containing rheumatoid factors were subjected to the assay. Interference by rheumatoid factors with sandwich immunoassay systems have been reported in [2].

Here, we describe a method to remove the interference by rheumatoid factors with the enzyme-linked sandwich immunoassay of insulin. Use of Fab′ fragments of antibody IgG fractions for preparing the antibody-immobilized solid phase and the antibody–enzyme complex is effective to remove interference by rheumatoid factors with the enzyme-linked sandwich immunoassay system.

2. Materials and methods

A porcine insulin solution, which was used in most of the experiments, was from Novo Industri A/S (Actrapid, 40 U/ml) and crystalline bovine insulin was from Fluka AG (23 U/mg). Guinea pig (anti-bovine insulin) serum was from Miles Labs (1.2 mU insulin bound/μl). Immunoglobulin G (IgG) fractions and their $F(ab')_2$ fragments of the antiserum were prepared as in [3,4]. Rheumatoid factors in sera were assayed with the human γ-globulin-immobilized latex particles (RA-test, from Hyland), and sera showed the agglutination at > 80-fold dilution with a saline, were used as samples containing rheumatoid factors (RF-positive serum).

2.1. *Guinea pig (anti-insulin)Fab′-β-D-galactosidase complex*

The $F(ab')_2$ fragments from the anti-insulin serum were reduced with 2-mercaptoethylamine and coupled to β-D-galactosidase from *E. coli* (Boehringer Mannheim) by use of *N,N′-o*-phenylenedimaleimide (Aldrich Chem. Co.) as in [4]. The amounts of the complex are expressed as units of enzyme activity, and the unit of activity is defined as in [1].

2.2. *Silicone rubber-immobilized anti-insulin (IgG or Fab′)*

The IgG fractions or their Fab′ fragments of anti-insulin serum were immobilized on silicone rubber (string, 3 mm diam. from Sanko Plastic Co., Osaka, cut into 4 mm lengths) as in [5]. The Fab′ fragments were prepared by reducing $F(ab')_2$ fragments with 15 mM 2-mercaptoethylamine. Sulfhydryl groups of the Fab′ fragments were blocked with *N*-ethylmaleimide. The anti-insulin-immobilized silicone rubber pieces were stored in buffer A (0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin (fraction V, Armour Pharmaceut. Co.) and 0.1% NaN_3) at 4°C for > 1 week before use.

2.3. *Immunoassay procedure*

A piece of the silicone rubber with immobilized antibodies (IgG or Fab′) was incubated in duplicate

with various amounts of insulin, or 10–100 μl of human serum 0.5 ml final vol. buffer G (0.01 M sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin, 0.5% gelatin (Difco Labs) and 0.1% NaN_3). After incubation at 30°C for 3 h with shaking, the reaction medium was discarded by aspiration, and each piece was washed successively with 1 ml buffer G and 1 ml buffer A in the test tube. The piece was then incubated at 4°C overnight with 3 munits of the (anti-insulin)Fab′–β-D-galactosidase complex in 0.2 ml buffer A. Each piece was washed twice with buffer A and the enzyme activity bound was assayed in buffer A as in [5] by use of 4-methylumbelliferyl-β-D-galactoside (Sigma Chem. Co.) as the substrate.

3. Results

Representative standard curves for the sandwich immunoassay of insulin with the (anti-insulin)Fab′–β-D-galactosidase complex and the silicone rubber solid phase with immobilized antibodies (IgG or Fab′) are shown in fig.1. When the enzyme activity bound was plotted as a function of insulin quantity, a linear dose–response was obtained between 0.25 and 8 μU porcine insulin in both assay systems, although the enzyme activity bound on the Fab′–solid phase was ~60% of that on the IgG–solid phase. Almost the same results were obtained with a crystalline bovine insulin.

The effect of rheumatoid factors on the immunoassay was tested by adding 10–100 μl of the RF-positive or RF-negative serum to the assay mixture with 2 μU insulin. Both sera contained < 0.25 μU endogenous insulin/50 μl. As shown in fig.2, enzyme activity bound on the IgG–solid phase was inhibited sensitively by the serum containing rheumatoid factors, whereas that was not inhibited by RF-negative serum. A slight increment of enzyme activity bound is due to the endogenous insulin in the RF-negative serum. The decrease in enzyme activity bound in the presence of rheumatoid factors was not due to

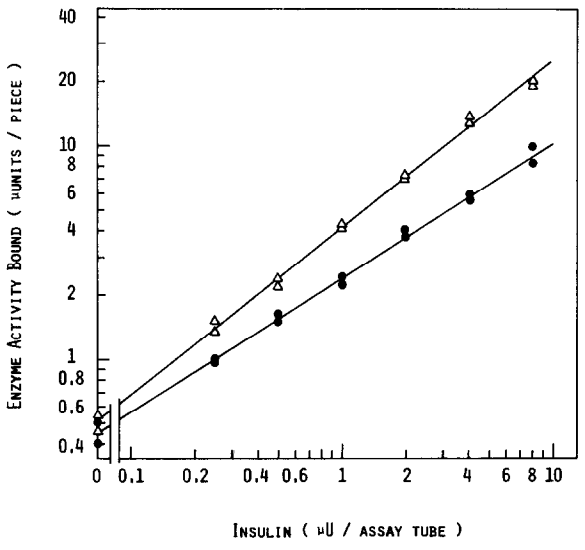

Fig.1. Standard curves for insulin estimation by enzyme-linked sandwich immunoassay. The assay was conducted with the (anti-insulin) Fab′–β-D-galactosidase complex and the (anti-insulin) IgG–silicone rubber (triangles) or the (anti-insulin) Fab′–silicone rubber (circles) as described in the text.

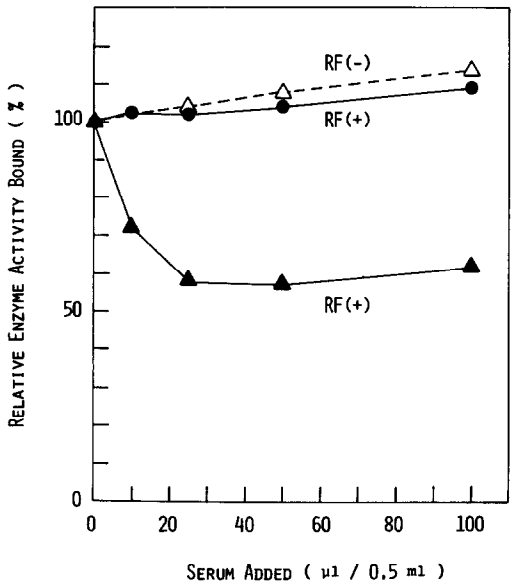

Fig.2. Effect of rheumatoid factors on the sandwich immunoassay systems for insulin. The (anti-insulin) IgG–silicone rubber (triangles) or the (anti-insulin) Fab′–silicone rubber (circles) was incubated with 2 μU insulin in the presence of the indicated volume of RF-positive or RF-negative serum, which contained < 0.25 μU insulin/50 μl. β-D-Galactosidase activities of 100% correspond to 7.01 μunits and 4.22 μunits in the assay with the IgG–solid phase, and with the Fab′–solid phase, respectively.

Table 1
Recovery of insulin added to serum samples

Rheumatoid factors in serum	No. of serum samples	Insulin recovered (μU)	
		a	b
Positive	10	0.63 ± 0.15[a]	0.97 ± 0.11[a]
Negative	9	0.96 ± 0.08	0.99 ± 0.17

[a] Means ± SD

The sandwich immunoassay of insulin was conducted with the (anti-insulin) IgG–silicone rubber (a) or with the (anti-insulin) Fab′–silicone rubber (b) as described in the text. Each serum sample of 50 μl was subjected to the assay in duplicate with or without 1 μU insulin, and the recovery of the added insulin was calculated from the corresponding standard curve

inactivation or inhibition of the enzyme protein, but to inhibition of the immunoreaction. However, the inhibition by rheumatoid factors was removed by using the Fab′–solid phase (fig.2).

To confirm the removal of interference by rheumatoid factors in the assay with the Fab′–solid phase, recovery experiments were carried out. Each serum sample of 50 μl was subjected to the immunoassay with or without 1 μU insulin, and the recovery of the added insulin was calculated from the corresponding standard curves. As shown in table 1, poor recovery was observed in the assay containing rheumatoid factors with the IgG–solid phase. However, the recovery was satisfactory in the assay with the Fab′–solid phase. Various amounts of insulin (0.5–6 μU) added with 50 μl serum containing rheumatoid factors were also completely recovered when assayed with the Fab′–solid phase (not shown). The recovery with RF-negative sera was satisfactory both in the assay with the IgG–solid phase and the Fab′–solid phase.

4. Discussion

As described [2], a drawback of the sandwich immunoassay system is observed when sera containing rheumatoid factors are assayed. Rheumatoid factors are antibodies with specificities for various antigenic determinants of Fc region of human IgG [6] and for the IgG of various other animal species [7]. Therefore, in the 'sandwich-type' immunoassay systems, rheumatoid factors react successively with the Fc region of some of the antibody IgG immobilized on a solid phase, and the Fc region of the labelled antibodies. Consequently, this interaction increases the binding of labelled antibodies on a solid phase, independently of the expected antigen–antibody reaction of the assay system.

The interference by rheumatoid factors appears in a different way in our assay system in which the antibody IgG–solid phase and the antibody Fab′–enzyme complex are used. Rheumatoid factors reacted with the antibody IgG fractions on a solid phase bring about a steric hindrance to the binding of the antigen to the antibodies immobilized on the solid phase, or/and that of labelled antibodies to the antigen trapped on the solid phase. Consequently, this interaction decreases the binding of labelled antibodies on a solid phase.

The inhibition of immunoreaction by rheumatoid factors was completely removed by use of antibody Fab′–solid phase as shown in the text. Theoretically, use of the antibody Fab′ fragments only for the solid phase is sufficient to remove the interference by rheumatoid factors in our assay procedure, because any serum components which did not react with the solid phase are washed out before the addition of the antibody–enzyme complex. Indeed, preliminary experiments of the immunoassay with the antibody IgG–β-D-galactosidase complex and the Fab′–solid phase showed little interference by rheumatoid factors, but the sensitivity of the assay with the IgG–enzyme complex was less than that with the Fab′–enzyme complex as in [4].

Inclusion of aggregated human IgG in the assay mixture for the assay of sera containing rheumatoid factors, as reported [2], did not remove the interference completely with our assay system.

Therefore, use of Fab′ (or Fab, fragments of IgG prepared with papain) fragments of antibody IgG is recommended for the determination of serum components in sera containing rheumatoid factors with the 'sandwich-type' immunoassay systems.

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